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UDC: 616.31+616.36:577.171.5]-092.9 **DOI: 10.18413/2313-8971-2016-2-2-41-54**

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MELATONIN ATTENUATES THE STRESS-INDUCED DISORDERS IN PARODONTIUM AND LIVER

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Abstract
The results of investigation illustrate the lipid peroxidation activation in different tissues under the stress influence. The obtained results convince the different resistance of animals to stress. The decrease of free and bound oxyprolin content, as well as glycosaminoglycans, which are the main components of connective tissue of gingiva, after prolonged stress has been established. Stress causes the development of structural and functional disorders in the liver tissue. Melatonin is effective to inhibit the stress-induced changes in liver and parodontium. The higher efficiency of melatonin at the dose 1,0 mg/kg over 0,2 mg/kg was established. The obtained results open the prospects of the using of melatonin and its artificial analogues for pharmacological treatment of stress-induced disorders in parodontium and liver.
Key words: stress, parodontium, liver, lipid peroxidation, melatonin.

Introduction

It's known the stress causes disturbances in blood flow, cell hypoxia, prooxidant-antioxidant disbalance and the formation of active forms of oxygen [1]. The numerous data show the development of structural and functional disorders in parodontium under the stress influence [2]. The development of lipid peroxidation (LPO) accompanied with chronic stress provokes the morphological and biochemical disorders formation in parodontium [3, 4].

It's shown that liver is a target organ for stress. Stress-induced disorders of liver tissue manifest themselves by dystrophy of hepatocytes, the dilation of intrahepatic vessels and the disorders of hepatocytes regeneration [5]. The stress development is accompanied with metabolism disturbances and this indicates to the formation of functional disorders in liver, because it has a very important role in metabolism regulation [6].

The above-mentioned data make actual the investigation of the prevention of stress-induced injuries of parodontium and liver.

Melatonin is one of the main substances of the stress-limiting system of a human organism. The hormone decreases stress-induced activation of hypothalamo-pituitary-adrenal system [7], has an anti-inflammatory, analgesic [8, 9] and strong antioxidant effect, including scavenger of free radicals, reinforcement of antioxidant enzymes activity, potentiation of the action of another endogenous antioxidant [10, 11]. The changes in melatonin content in blood were established in patients suffering from parodontium diseases [12]. The treatment of parodontium disturbances influences the melatonin concentration in salivary glands [4]. Nevertheless, the effects of melatonin in parodontium of animals exposed to stress are not sufficient.

The stress-induced LPO is one of the most important mechanisms of liver injury. Because of it the using of melatonin, manifesting of strong antioxidant effect can be effective for prevention of stress-induced structural and functional disturbances in liver.

The purpose of this report is the investigation of melatonin influence on stress-induced disorders in parodontium and liver of animals characterizing by different stress-resistance.

Materials and methods

The study was conducted on 680 sexually mature male albino rats Wistar, weighting 180-220 g, obtained from Stolbovaya filial of National Center of Biomedical Technologies of Federal Medicobiological Agency (NCBMT FMA) (Moscow, Russia). The animals were housed in groups of five rats per cage. The rats were acclimatized for two weeks before using them. The rats were maintained under control conditions: temperature 23+-2^{o}C, light regime: 12 hours light: 12 hours darkness. The animals were provided with standard diet containing pelleted food and water *ad libitum*. The experimental protocol was based on the principals of Convention for defense of vertebrates animals, used for experimental and other purposes (Strasbourg, France). The University Animal Ethic Committee approved this experimental protocol.

To separate stress-resistant and stress-nonresistant rats "open field" test was used [13]. "Open field" is a round ground (diameter 1 m), divided into 32 sectors by radiuses and circumferences. 1 meter over the center of the field an electric bulb with routes 60Wt was installed.

Stress-resistant animals are characterized by: a short latent period of the first movement beginning (less than 3 s), high horizontal (more than 80 crossed sectors) and vertical (more than 10 sets), active grooming (more than 10 s), high investigated activity (more than 5 peeps in holes), low autonomic balance (0-1 boluses). Stress-nonresistant animals have opposite characteristics: a long latent period of the first movement beginning (more than 10 s), low horizontal (less than 40 crossed sectors) and vertical (less than 8 sets), rare grooming (less than 4 s), low investigated activity (less than peeps in holes), high autonomic balance (more than 2 boluses).

96 stress-resistant and 96 stress-nonresistant rats were chosen. Animals which are not related to these two groups were excluded from the experiment.

The prolonged decreased mobility stress model was used, in which the animal was set to a special box of small volume to prevent any movements without food and water for 6 hours during 12 days [14].

Acute 6-hour immobilization stress was produced by the way of animal fixation on the special table. The animals were withdrawn from the experiment 39 hours, 4 and 7 days after the end of immobilization. The choice of these periods is determined by the literature data that maximal changes in the internal organs are developed at the end of alarm stage (39 hours after stress), and compensatory reactions in the injured organs are manifested obviously at the beginning of resistance stage (4 days after stress) and 7 days after stress [5].

Melatonin (Aldrich-Sigma, USA) was used in the experiments. Melatonin preparation was dissolved in 1% ethanol solution. The rats of melatonin-treated groups were given melatonin at doses 0,2 or 1,0 mg per kg of body weight intraperitoneally each day within 7 days after prolonged decreased mobility stress modeling. Control rats received a similar amount of 1% ethanol solution in the same manner.

The animals exposed to acute immobilization stress were given melatonin at doses 0,2 or 1,0 mg per kg of body weight intraperitoneally each day within 5 days after the experiment beginning. The rats sacrificed 39 after stress were given 2 preparation injections, 4 days – 4 injections.

On the completion of the experiment the rats were sacrificed with ether over dosage.

39 hours, 4 and 7 days after acute immobilization stress modeling and 12 days after prolonged decreased mobility stress, animals of each group were anaesthetized by ether. Blood was collected directly from the heart in heparinized vials. Plasma was separated by centrifuging the blood at 3000 rpm at 4^{o}C for 15 min. The supernatant was used for estimation of LPO metabolites malonic dialdehyde (MDA), acylhydroperoxides (AHP) concentrations and antioxidant enzymes activity: superoxiddismutase (SOD) and catalase, as well as the content of albumin, plasma protein, cholesterol, triglycerides (TG), non-esterified fatty acids (NEFA), lipoproteins of very low density (LPVLD), lipoproteins of low density (LPLD), lipoproteins of high density (LPHD), alanine aminotransferase (AlAT) and aspartate aminotransferase (AsAT) by traditional methods [15].

The rats were sacrificed then by ether over dosage, and mandible and a piece of liver tissue were taken for further investigations. Gingival tissue of a half of mandible (100 mg) and a piece of liver tissue (100 mg) were homogenized with tissue homogenizer in 1 ml of 0,025 mM tris-HCl buffer (pH=7,4). The free supernatant of gingiva tissue was obtained by centrifugation at 5000 rpm at 4^{o}C for 20 min and used for estimation of MDA, AHP, free and bound oxyprolin, GAG, catalase activities.

The free supernatant of liver tissue was obtained by centrifugation at 5000 rpm at 4^{o}C for 20 min and used for estimation of MDA, AHP, SOD and catalase activities.

The level of MDA was measured using the mixture of thiobarbituric and acetic_acids, then optic density was measured in 532nm and MDA concentration was counted in mcmol/g in gingival and liver tissues and mcmol/ml in plasma [16]. The level of AHP in specimen was detected in heptan in 233 nm and expressed in special units [16]. SOD activity was detected by method based on the determination of the degree of inhibition of nitroblue tetrasolium reduction reaction. SOD activity was expressed in special units [17]. Catalase activity was detected by method based on the capability of hydrogen peroxide to form stained complex with molibden salts [18]. Catalase activity was counted in mcat/g of tissue and mcat/l in plasma. The content of free and bound oxyprolin was detected by method based on oxyprolin oxidation by chloramin B [19]. Clycosaminoglycans (GAG) content was detected by using of trichoracetic acid and carbonyl reaction [20].

For morphological investigation of parodontium the fragment of mandible was fixed in 10% neutral formalin with subsequent decalcination. The slides from paraffin blocks were prepared (thickness was 5-6 mcm) and then stained by hemotoxilin+eosin or according to Van Gison. The histological and planimetry analyses were fulfilled.

Biochemical analyses were presented as the mean +-standard error of means (S.E.M). Comparisons were

made between naïve, control (stressed) and stressed melatonin-treated groups on computer using "Statistica 6.0". A "P" value of 0,05 was selected as a criterion for statistically significant differences.

Results

The edema of parodontium mucosa, especially in the lateral parts, the hyperemia of gingiva and the dilation of vessels were observed in stress-resistant and stress-nonresistant rats exposed to prolonged decreased mobility stress. These changes were more expressed in stress-nonresistant rats, and teeth coating was revealed in them too.

The increase of thickness of corneal layer was observed in animals of both groups exposed to chronic stress. But the thickness of epithelium is significantly decreased compared to naïve animals due to prickly and granular layers destruction. The dystrophy of epithelium is observed mainly in prickly and granular layers. The loosening of epithelium develops as the result of edema. There are a lot of macrophages and neutrophils in the epithelium layer. The dilation of vessels and increase of fibroblasts amount are observed in propria lamina. It is infiltrated by neutrophils and macrophages. The decrease of the thickness of cortical laminas and bone trabecules of spongy tissue is revealed in peripheral regions. All the indicated changes are obviously observed in stress-nonresistant animals, in which leukocyte infiltration and generalized thinning of parodontium bone tissue are more expressed (Picture 1).

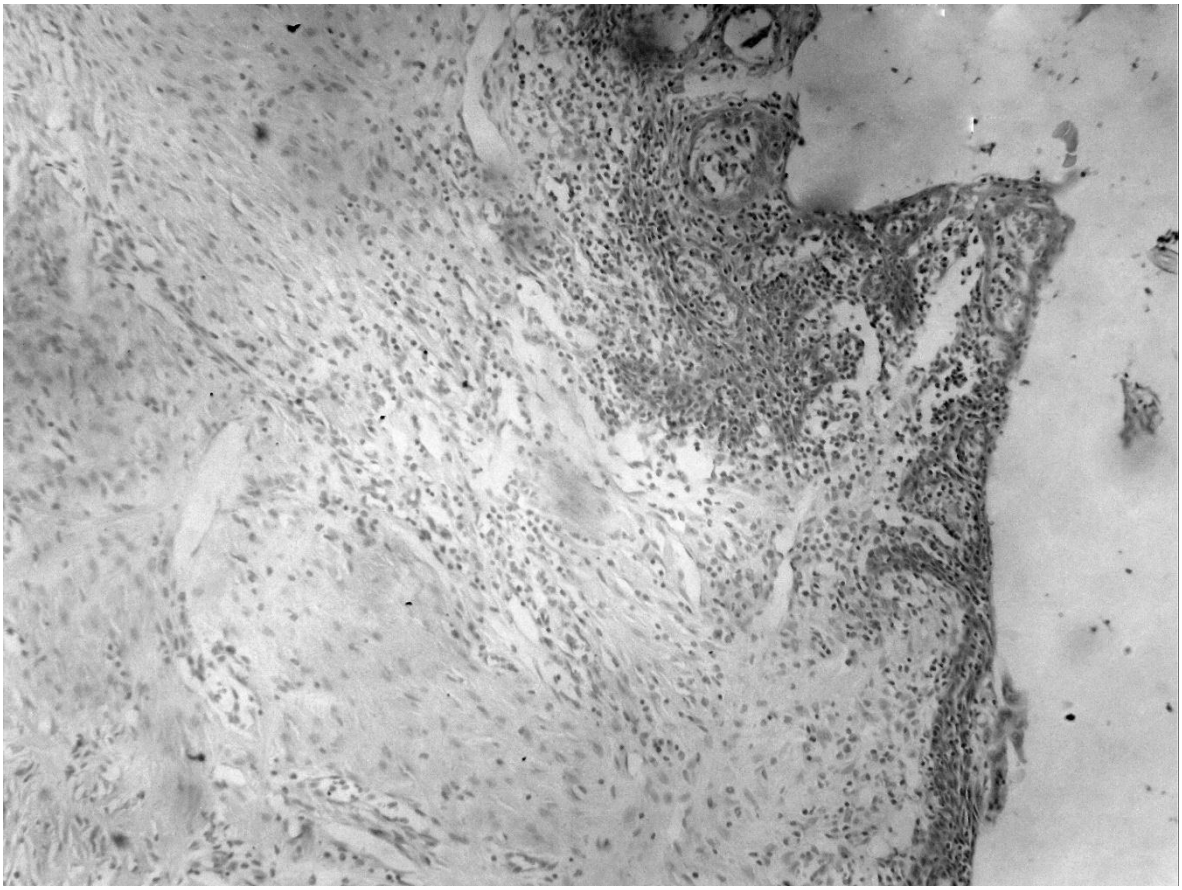

Figure 1. Stress-induced changes in parodontium of stress-nonresistant control animals: the decrease of epithelium thickness, edema, leukocyte infiltration of lamina propria. Hematoxilin&eosin. X 100.

The administration of melatonin to the animals of both groups caused the weakening of stress-induced parodontium disorders. Although hyperemia was present, but the edema of mucosa was absent, excepting some stress-nonresistant rats. Tooth coating was not determined.

The microscope examination revealed the increase of epithelium thickness, minor dystrophic changes in epithelium compared to the corresponding control group. The edema and the number of dilated vessels were significantly fewer in all the groups of melatonin-treated animals compared to control rats. The administration of melatonin prevented the stress-induced changes in the parodontium bone tissue. Cortical lamina and trabecules of spongy bones had almost normal thickness. The effects of the investigated preparation were more expressed at the dose of 1,0 mg per kg b.w (Picture 2).

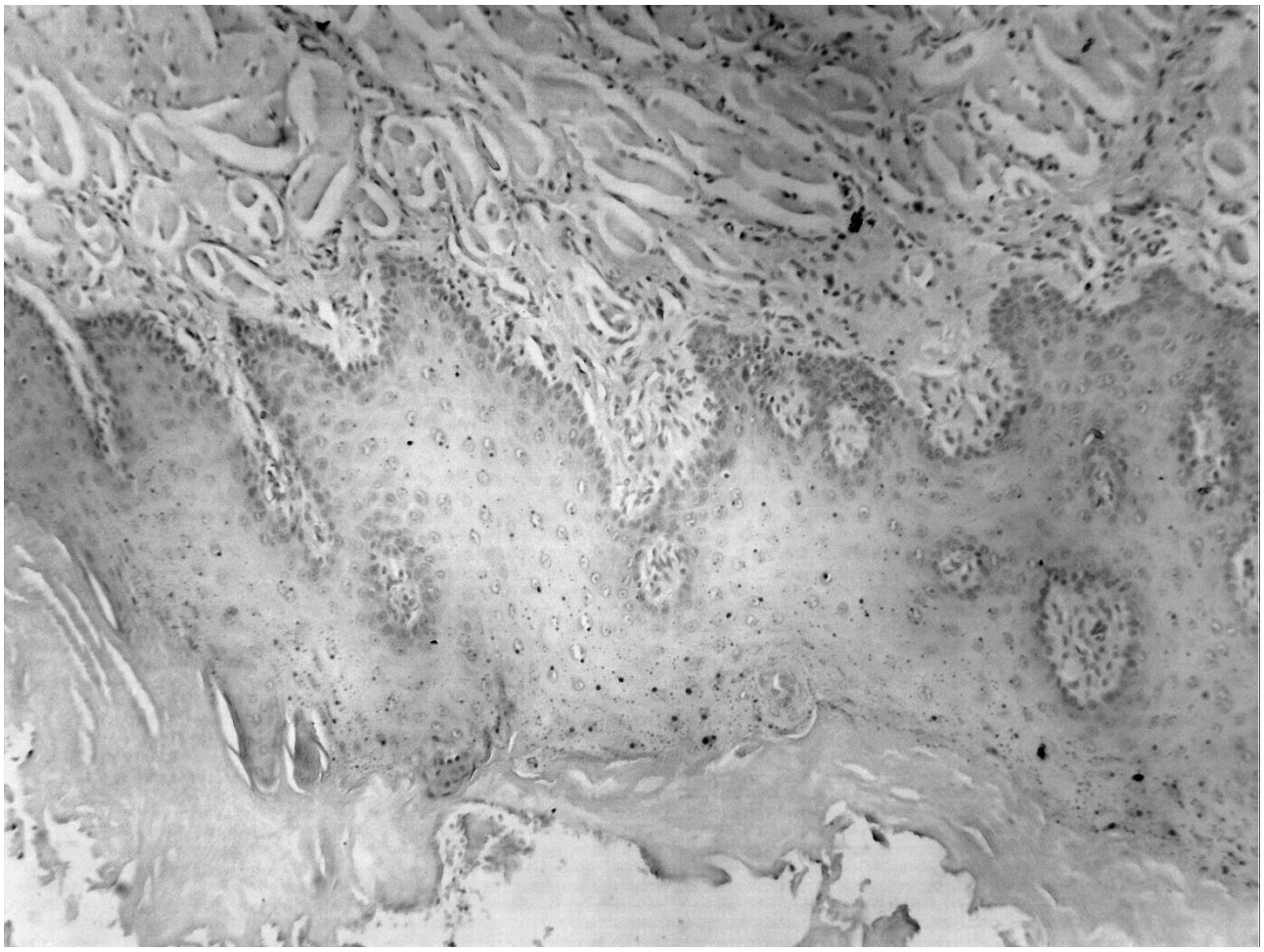

Figure 2. Stress-induced changes in parodontium of stress-nonresistant animals treated with melatonin at the dose 1,0 mg/kg: the decrease of epithelium thickness, edema, leukocyte infiltration of lamina propria are weakly expressed. Hematoxilin&eosin. X 100.

MDA and AHP concentrations were increased in gingival tissue in both stress-resistant and stress-nonresistant rats after 12-days of decreased mobility stress. The drop of catalase activity was observed in stress-nonresistant animals, but not in stress-resistant ones. It has shown the decrease of free and bound oxyprolin and GAG in the gingival tissue of rats after stress. The obtained results are shown in Tables 1 and 2.

Table 1

The influence of melatonin on the content of bound oxyprolin, glycoaminoglycans, malonic dialdehyde, acylhydroperoxides and catalase activity in gingival tissue of stress-resistant rats after chronic stress (M+-m; n=8)

Index Group	Content of free oxyprolin in gingiva, mmol/g	Content of bound oxyprolin in gingiva, mmol/g	Content of glycosaminoglycans in gingiva, mg/g	Content of malonic dialdehyde in gingiva, mcmol/g	Content of acyhydroperoxides in gingiva, s.u.	Catalase activity in gingiva, mcat/g
Naive (intact)	5,80±0,30	5,86±0,26	7,43±0,30	13,28±0,39	3,83±0,25	27,29±0,55
Control (stressed)	5,19±0,23	5,49±0,28	7,15±0,33	16,14±0,60^{xx}	5,30±0,39^{x}	26,55±0,49
Stress+melatonin at the dose 1 mg/kg	5,74±0,30	5,88±0,27	7,09±0,35	14,21±0,38*	3,90±0,29*	23,69±0,36**
Stress+melatonin at the dose 0,2 mg/kg	5,71±0,31	5,98±0,32	7,00±0,48	15,04±0,57	4,84±0,38	26,49±0,60

Note: ^{x} – at $p<0.05$ as compared to intact rats, ^{xx} – at $p<0.01$ as compared to intact rats. * - at $p<0.05$ as compared to control rats, ** – at $p<0.01$ as compared to control rats.

The injections of melatonin solution at the dose 1,0 mg per kg of b.w. within 7 days inhibited the lipid peroxidation in gingival tissue in both investigated groups of rats, that was manifested with the reduction of MDA and AHP concentrations. The preparation administration had opposite effects on catalase activity in gingiva of rats after stress. The decrease of this enzyme activity was observed in stress-resistant rats and the increase - in stress-nonresistant. Besides the increase of free and bound oxyprolin and glycosaminoglycans was detected only after stress in gingiva of stress-nonresistant rats, which received hormone injections.

Table 2

The influence of melatonin on the content of bound oxyprolin, glycoaminoglycans, malonic dialdehyde, acylhydroperoxides and catalase activity in gingival tissue of stress-nonresistant rats after chronic stress (M+-m; n=8)

Index / Group	Content of free oxyprolin in gingiva, mmol/g	Content of bound oxyprolin in gingiva, mmol/g	Content of glycosaminoglycans in gingiva, mg/g	Content of malonic dialdehyde in gingiva, mcmol/g	Content of acyhydroperoxides in gingiva, s.u.	Catalase activity in gingiva, mcat/g
Naive (intact)	6,21±0,25	6,59±0,29	7,38±0,40	14,06±0,41	3,96±0,19	26,24±0,66
Control (stressed)	4,24±0,38	4,46±0,	5,54±0,27^{xx}	19,63±0,73^{xxx}	5,65±0,37^{xx}	22,69±0,67^{xx}
Stress+melatonin at the dose 1,0 mg/kg	5,51±0,32*	5,78±0,34*	6,84±0,31*	16,64±0,79*	4,50±0,31*	25,81±0,77*
Stress+melatonin at the dose 0,2 mg/kg	4,59±0,27	5,34±0,38	6,56±0,35*	17,05±0,73*	4,53±0,40*	25,61±0,54*

Note: ^{xx} – at p<0.01 as compared to intact rats, ^{xxx} – at p<0.001 as compared to intact rats. * - at p<0.05 as compared to control rats.

The administration of melatonin at the dose 0,2 mg per kg of b.w. had antioxidant effect in stress-nonresistant rats after stress, but not in stress-resistant ones. It was established that MDA and AHP, catalase activity decreased in them. The injections of preparation at small dose caused the increase of GAG content, but not free and bound oxyprolin in gingiva of stress-nonresistant rats after stress.

In both groups of rats, exposed to 6-hour immobilization stress, the decrease of the thickness of granular layer was determined 39 hours and 4 days after stress modeling. In stress-nonresistant animals, the rare dystrophy of epithelial cells was considered. Infiltration of epithelial layer by macrophages and neutrophils is not evident. In some preparations the decrease of the thickness of cortical laminas and bone trabecules are revealed. The effects of melatonin on the morphological changes in parodontium in the both investigated groups are not determined.

The modeling of 6-hour immobilization stress causes the shift of prooxidant-antioxidant balance in the mucosa of parodontium of stress-resistant animals 39 hours after the experiment beginning, that manifested by the increase of the content of LPO intermediate metabolites – AHP (by 24,9%, p<0,05), there was no increase of MDA concentration and changes in catalase activity.

The arising of LPO metabolites was observed in stress-nonresistant rats 39 hours after 6-hour immobilization stress: AHP – by 44,1% (p<0,01) and MDA – by 18,7% (p<0,05). The catalase activity was not changed.

There were no differences between rats, exposed to stress, and naïve ones of corresponding typological group on the fourth and the seventh days after the experiment beginning.

The administration of melatonin at the dose 1,0 mg per kg b.w. caused the suppression of LPO, manifested by the decrease of AHP content by 10,3% in stress-resistant rats, exposed to 6-hour immobilization stress compared to control animals. The injections of melatonin at the dose 0,2 mg per kg had no influence on LPO.

The decrease of LPO in parodontium mucosa is observed in melatonin-treated (dose is 1,0 mg per kg b.w.) stress-nonresistant rats 39 hours after stress modeling. MDA concentration was less in them by 16,4% (p<0,05) compared to control ones. The normal level of LPO and catalase activity was revealed in melatonin-treated animals, exposed to immobilization stress, on the fourth and the seventh days after stress modeling.

There were no changes in the content of collagen and GAG in parodontium in the rats, exposed to 6-hour immobilization stress.

The modeling of prolonged decreased mobility stress caused the following changes in liver tissue microstructure in stress-resistant animals: the decrease of normal hepatocytes number by 11,0% (p<0,001), the increase of dystrophic cells by 70,4% (p<0,001), the increase of the specific area of

dystrophic regions of cytoplasm in 4,4 times (p<0,001) compared to naïve animals (Table 3). The sum volume of sinusoids was increased by 60,5% (p<0,001), that indicates to disturbances in liver tissue microcirculation.

Table 3

The influence of melatonin on morphological changes in liver tissue after prolonged decreased mobility stress (M+-m; n=8)

Index / Group	The share of normal hepatocytes, %	The share of dystrophic hepatocytes, %	Specific area of dystrophically changed cytoplasm regions, sp. un.	The overage volume of nuclei, sp. un.	The share of binuclear hepatocytes, %	The share of nuclei containing one nucleolus, %	Sum volume of sinusoids, %	Sum volume of stroma, sp. un.
Stress-resistant naïve (intact)	86,5±0,6	13,6±0,6	0,05±0,01	0,23±0,01	18,5±0,9	83,8±0,7	8,6±0,5	0,18±0,01
Stress-resistant stressed	77,0±1,0^{xxx}	23,0±1,0$^{x}_{xx}$	0,22±0,02^{xxx}	0,27±0,01^{xx}	25,9±1,1^{xxx}	73,9±1,5^{xxx}	13,8±0,5^{xx}	0,20±0,01
Stress-resistant stressed + melatonin at the dose 0,2 mg/kg	76,5±1,4	23,5±1,4	0,19±0,02	0,26±0,01	26,1±1,1	72,9±1,3	12,0±0,4*	0,20±0,01
Stress-resistant stressed + melatonin at the dose 1,0 mg/kg	80,9±1,0*	19,1±1,0*	0,16±0,01*	0,28±0,01	29,5±0,7*	65,8±1,4**	11,6±0,5*	0,20±0,01
Stress-nonresistant naïve (intact)	84,8±1,2	15,2±1,2	0,06±0,01	0,21±0,01	19,6±0,8	85,3±1,1	10,5±0,4	0,19±0,01
Stress-nonresistant stressed	51,3±1,3^{xxx}	48,7±1,3$^{x}_{xx}$	0,48±0,04^{xxx}	0,26±0,01	23,3±1,0^{x}	72,8±1,1^{xxx}	19,3±0,7^{xxx}	0,21±0,01
Stress-nonresistant stressed + melatonin at the dose 0,2 mg/kg	69,9±1,5***	30,1±1,5***	0,36±0,03*	0,24±0,01	26,3±0,8*	70,9±1,4	18,6±0,5	0,20±0,01
Stress-nonresistant stressed + melatonin at the dose 1,0 mg/kg	73,3±1,9***	26,7±1,9***	0,32±0,03**	0,28±0,01	27,0±0,8*	67,6±1,5*	16,5±0,7*	0,20±0,01

Note: ^{x} – at p<0.05 as compared to intact rats, ^{xx} – at p<0.01 as compared to intact rats, ^{xxx} – at p<0.001 as compared to intact rats. * - at p<0.05 as compared to control rats, *** - at p<0.001 as compared to control rats.

The activation of reparative processes was observed simultaneously in liver tissue: the overage size of nuclei was increased by 17,4% (p<0,05), the share of two-nuclear hepatocytes arose by 40,0% (p<0,01), the share of nuclei containing one nucleolus was decreased by 11,9% (p<0,001) (Picture 3).

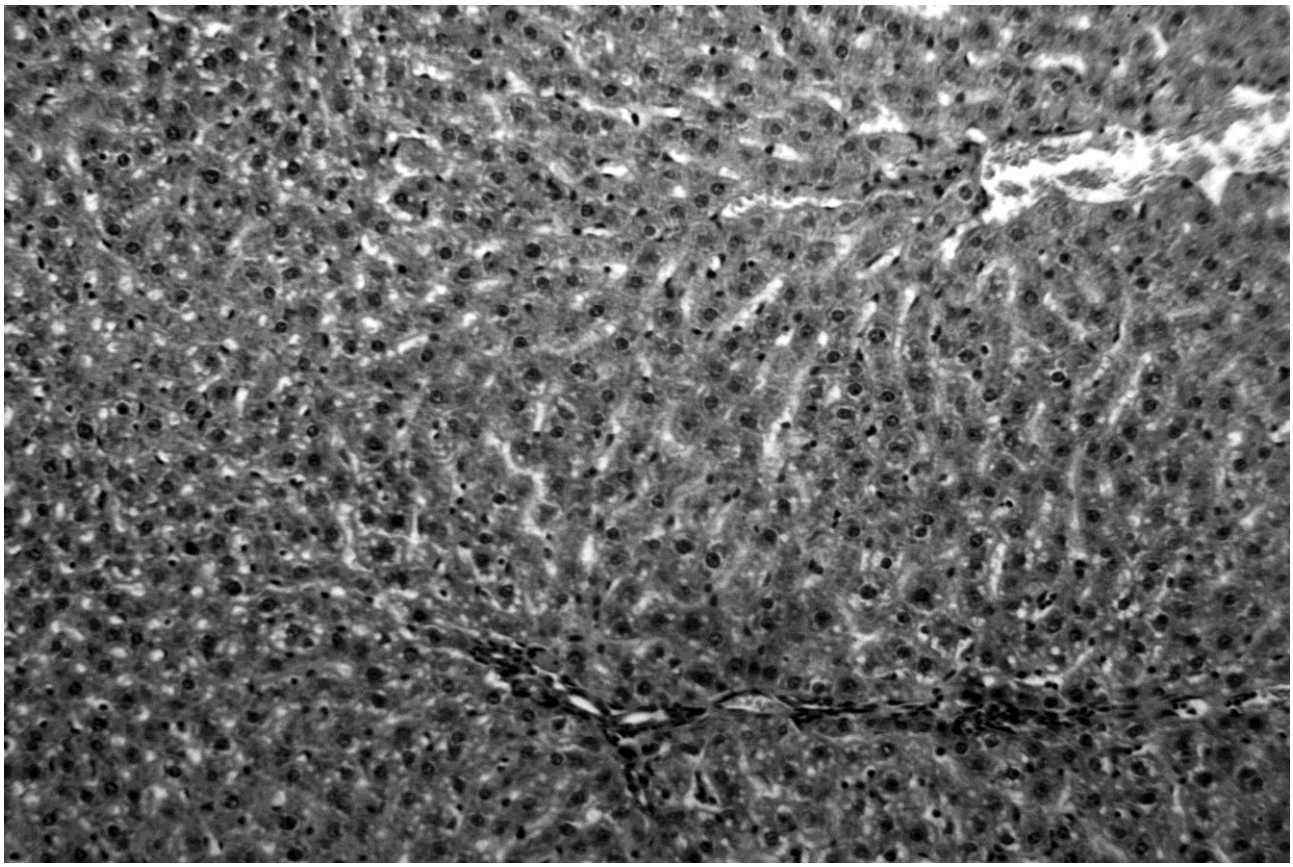

Figure 3. Dystrophy of hepatocytes of stress-resistant rats, exposed to prolonged decreased mobility stress. The number of dystrophic cell is low. Hematoxilin&eosin. X 140.

The modeling of prolonged decreased mobility stress to stress-nonresistant rats caused a significant increase of dystrophic hepatocytes by 3,2 times ($p<0,001$) and the decrease of normal hepatocytes amount by 39,5% ($p<0,001$). The increase of the specific area of dystrophically changed cytoplasm regions by 7,3 times ($p<0,001$) was observed in them compared to naïve animals. The sum volume of sinusoids was increased by 96,9% ($p<0,001$) (Picture 4)

The activation of reparative processes in this group manifested itself by increase of overage size of nuclei by 23,8% ($p<0,01$), the decrease of the share of nuclei containing one nucleolus by 8,7% ($p<0,01$).

Pharmacological effects of melatonin at the dose 1,0 mg per kg in stress-nonresistant rats include the significant decrease of the dystrophic hepatocytes number by 45,2% ($p<0,001$), the increase of normal hepatocytes by 42,9% ($p<0,001$), the decrease of the specific area of dystrophically changes cytoplasm regions by 31,8% ($p<0,05$), the drop of the sum volume of sinusoids by 14,5% ($p<0,05$), the arising of the share two-nuclear hepatocytes by 19,4% ($p<0,01$) and the decrease of the share of nuclei containing one nucleolus by 11,8% ($p<0,01$). The effects of melatonin at the dose 0,2 mg per kg has the same direction but less.

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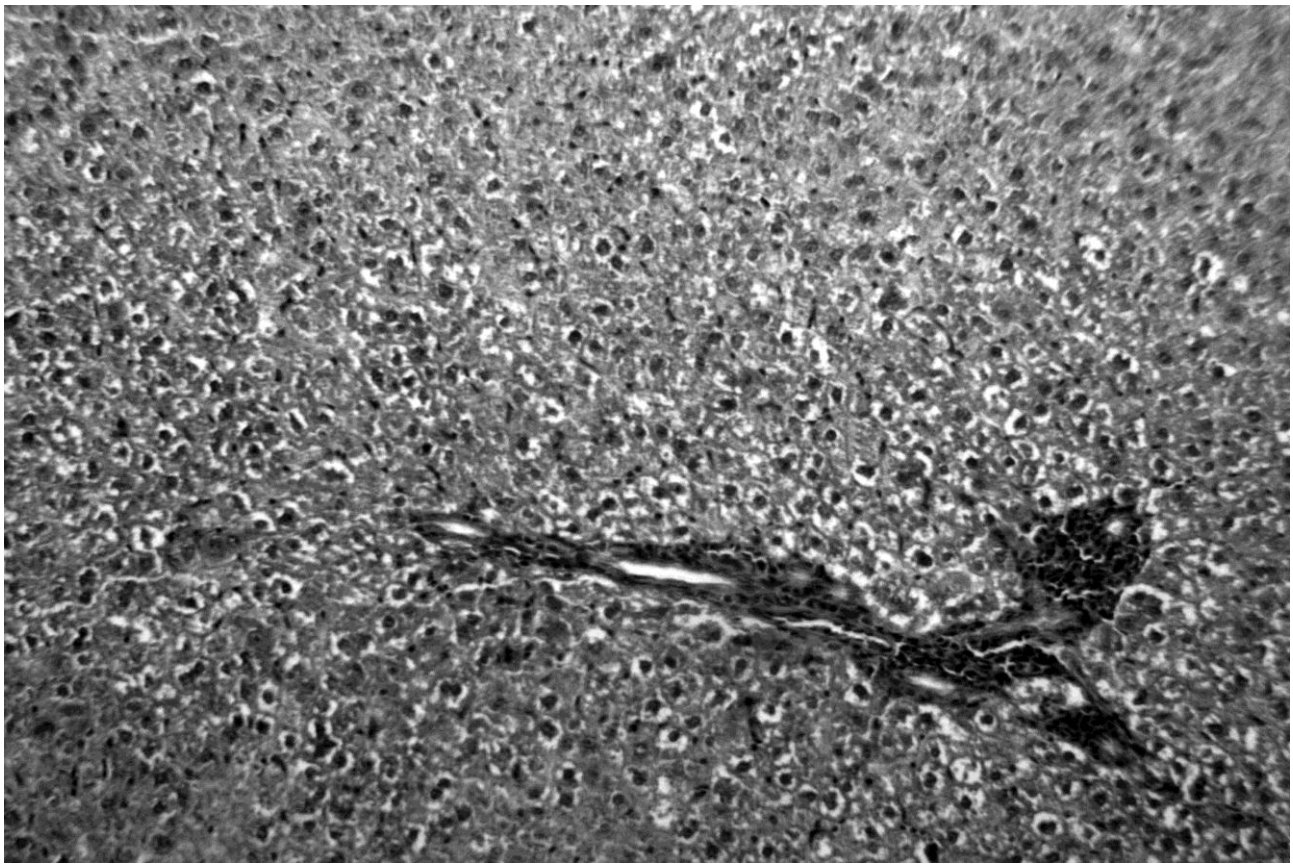

Figure 4. Dystrophy of hepatocytes of stress-nonresistant rats, exposed to prolonged decreased mobility stress. The number of dystrophic cell is high and heterogeneity of cytoplasm is expressed. Hematoxilin&eosin. X 140

The modeling of 6-hour immobilization stress caused significant increase of dystrophic hepatocytes, the decrease of normal hepatocytes number, the increase of specific area of dystrophically changed cytoplasm regions, the increase of sum volume of sinusoids in stress-resistant and stress-nonresistant rats 39 hours and 4 days after the experiment beginning. The activation of reparative processes in liver parenchyma becomes evident on the fourth day of the experiment and manifested itself by the increase of the share of two-nuclear hepatocytes and the decrease of the share of nuclei containing one nucleolus. There were no differences in all the investigated indices between rats, exposed to 6-hour immobilization stress, and naïve ones 7 days after stress modeling.

The administration of melatonin at the doses 1,0 and 0,2 mg per kg caused the decrease of the share of nuclei containing one nucleolus in stress-resistant rats 39 hours after immobilization (by 5,3% and 6,5% correspondently, $p<0,05$). Stress-limiting effects of melatonin at the dose 1,0 mg per kg are the most evident on the fourth day of the experiment: the decrease of dystrophic hepatocytes by 29,2% ($p<0,01$), the increase of normal hepatocytes number by 6,2% ($p<0,01$), the decrease of the specific area of dystrophically changed cytoplasm regions by 36,4% ($p<0,05$), the decrease of sum volume of sinusoids by 19,8% ($p<0,05$) compared to control animals. The activation of reparative processes in the liver tissue manifested itself by less share of nuclei containing one nucleolus (by 4,5%, $p<0,01$). The animals, treated by melatonin at the dose 0,2 mg per kg b.w., were characterized by decreased sum volume of sinusoids (by 16,5%, $p<0,05$). The stress-limiting effect of melatonin at the dose 1,0 mg per kg was evident on the seventh day too.

The significant decrease of the specific area of dystrophically changed cytoplasm regions by 33,3% and 38,1% correspondently is revealed in stress-nonresistant rats compared to control group 39 hours after the experiment ($p<0,001$). Melatonin-treated stress-nonresistant animals (dose 1,0 mg per kg b.w.) showed the significant decrease of dystrophic hepatocytes number (by 22,3%, $p<0,01$) and increase of normal hepatocytes amount (by 4,9%, $p<0,01$). The activation of reparative processes in liver tissue of melatonin-treated rats, exposed to 6-hour immobilization stress, is confirmed by the significant increase of the share of binuclear hepatocytes (by

9,1%, p<0,05) and the decrease of the share of nuclei containing one nucleolus (by 4,2%, p<0,05).

The administration of preparation at the dose 0,2 mg per kg didn't affect the investigated parameters in stress-nonresistant animals on the fourth day of the experiment.

The administration of melatonin at the doses 1,0 and 0,2 mg per kg b.w. increased the share of binuclear hepatocytes in stress-nonresistant animals on the seventh day of the experiment compared to the control group (p<0,01).

The modeling of prolonged decreased mobility stress is accompanied with the increase of the content of LPO metabolites in liver tissue (Table 4). Besides, the activity of antioxidant enzymes was inhibited, that indicates to the suppression of adaptation processes in the organ.

Table 4

Influence of melatonin on the content of lipid peroxidation metabolites and antioxidant enzymes activity in liver tissue in prolonged decreased mobility stress (M+-m; n=8)

Indices / Group	Content of malonic dialdehyde, mcmol/l	Content of acylhydroperoxidesides, su	Superoxidismutase activity, su	Catalase activity, mcat/ml
Naïve (intact)	32,1±1,5	4,4±0,3	54,5±1,9	48,4±1,7
Control (stressed)	48,4±2,4^{xxx}	9,6±0,6^{xxx}	34,2±1,3^{xxx}	28,4±1,8^{xx}
Stressed + melatonin at the dose 0,2 mg/kg	41,0±1,7*	6,2±0,4**	40,7±1,4**	38,6±1,4**
Stressed + melatonin at the dose 1,0 mg/kg	38,6±1,7**	6,1±0,4**	46,8±1,5***	40,5±1,5***

Note: ^{xx} – at p<0.01as compared to intact rats, ^{xxx} – at p<0.001as compared to intact rats, * - at p<0.05 as compared to control rats, ** - at p<0.01 as compared to control rats, *** - at p<0.001 as compared to control rats.

Intraperitoneal injections of melatonin at the dose 1,0 mg per kg b.w. within 7 day of stress modeling caused the decrease of LPO metabolites concentration: MDA – by 20,2% (p<0,01) and AHP – by 37,2% (p<0,01) compared to control group. The activity of investigated enzymes was increased: SOD – by 36,8% (p<0,01) and catalase – by 42,5% (p<0,001). The effect of melatonin at the dose 0,2 mg per kg were the same but its expression was less.

The development of acute immobilization stress is accompanied with prooxidant-antioxidant balance disorders, LPO activation in the liver tissue. The activity of both antioxidant enzymes in rats, exposed to acute immobilization stress, was suppressed in liver tissue.

The pharmacological antioxidant effect of melatonin at the dose 1,0 mg per kg was significantly more expressed. The concentrations of MDA and AHP were less compared to control group 39 hours after stress modeling and besides these were almost the same to MDA and AHP value in naïve animals 4 days after the experiment beginning.

There were no differences between the concentrations of LPO metabolites, AlAT, AsAT, general protein, albumin, cholesterol, TG, NEFA, LPVLD, LPLD, LPHD of stress-resistant and stress-nonresistant animals in our investigation, because of it the data of biochemical investigation in plasma are presented without the separation of typological groups.

The development of acute 6-hour immobilization stress causes the increase of AlAT and AsAT content, the decrease of plasma protein content 39 hours after immobilization stress. Albumin concentration was decreased compared to naïve animals by 3,5% (p<0,05) 4 and 7 days after stress modeling. The administration of melatonin at the dose 1,0 mg per kg b.w. caused the decrease of AlAT concentration by 20,0% and AsAT one by 22,2% (p<0,05) compared to control group 39 hours after stress. Melatonin injections had no effect on the concentration of general protein and albumin in animals, exposed to 6-hour immobilization stress (Table 5).

Table 5

The influence of melatonin on the concentration of AsAT, AlAT, general proteins, albumins, glucose in the plasma of animals, exposed to 6-hour immobilization stress (M+-m; n=8)

Index / Group	The period after immobilization	Aspartate aminotransferase, un/l	Alanine aminotransferase, un/l	Albumin, g/l	General protein, g/l	Glucose, mrmol/l
Naive (intact) group		196,6±12,1	55,5±3,5	31,0±0,2	67,9±1,2	8,4±0,2
Control (stressed) group	39 h.	499,9±32,6^{xxx}	166,6±12,2^{xxx}	30,7±0,4	64,3±0,6^{x}	8,5±0,2
	4 days	215,1±21,9	55,5±5,9	29,9±0,4^{x}	69,8±1,0	8,9±0,2
	7 days	188,8±6,9	59,0±4,3	29,5±0,4^{x}	67,4±1,3	8,6±0,3
Stressed group + melatonin at the dose 0,2 mg/kg	39 h.	443,0±16,4^{xxx}	143,9±3,0^{xxx}	29,5±0,4^{x}	64,4±0,7^{x}	9,0±0,3
	4 days	217,3±14,6	59,6±5,6	29,4±0,5^{x}	68,6±1,1	9,1±0,3
	7 days	205,3±11,1	55,2±5,8	29,4±0,3	68,0±1,4	9,1±0,3
Stressed group + melatonin at the dose 1,0 mg/kg	39 h.	399,6±2,3*^{xxx}	129,5±3,9*^{xxx}	29,5±0,4^{x}	63,8±0,6	9,0±0,3
	4 days	185,9±15,0	62,8±5,6	29,8±03^{x}	66,5±1,4	8,6±0,3
	7 days	159,4±6,1	57,8±4,2	29,6±0,3^{x}	66,8±0,9	8,7±0,2

Note: ^{x} – at p<0.05as compared to intact rats, ^{xxx} – at p<0.001as compared to intact rats, * - at p<0.05 as compared to control rats.

The modeling of prolonged decreased mobility stress within 12 days caused the arising of AlAT and AsAT content in plasma by 2,6 and 3,6 times correspondently (p<0,001) (Table 6). The drop of albumin plasma concentration by 8,1% was revealed (p<0,01). The administration of melatonin at the doses 1,0 and 0,2 mg per kg b.w. resulted in significant decrease of AlAT and AsAT concentrations in plasma (p<0,01-0,001). Albumin content was significantly higher than in the control group (p<0,05-0,01).

The prolonged decreased mobility stress caused the disorders of prooxidant-antioxidant balance in blood plasma. The concentrations of LPO metabolites were increased: MDA – up to 92,6%, AHP – up to 134,1% (p<0,001) compared to naïve animals (Table 7). The activity of antioxidant enzymes in plasma was significantly suppressed: SOD – by 48,1%, catalase - by 28,2% (p<0,001).

Table 6

The influence of melatonin on the concentration of AsAT, AlAT, general proteins, albumins, glucose in the plasma of animals, exposed to prolonged decreased mobility stress (M+-m; n=8)

Index / Group	Aspartate aminotransferase, un/l	Alanine aminotransferase, un/l	Albumin, g/l	General protein, g/l	Glucose, mrmol/l
Naïve (intact) group	203,3±8,7	54,5±3,2	29,7±0,4	67,1±1,8	8,5±0,2
Control (stressed) group	536,6±15,5^{xxx}	198,3±8,7^{xxx}	27,3±0,4^{xx}	63,3±0,9	8,5±0,2
Stressed group + melatonin at the dose 0,2 mg/kg	479,3±18,4**	153,0±7,8*	29,4±0,4**	67,8±1,2	8,8±0,2
Stressed group + melatonin at the dose 1,0 mg/kg	421,8±18,2***	134,4±7,3**	29,3±0,5*	67,5±1,1	8,7±0,3

Note: ^{xx} – at p<0.01 as compared to intact rats, ^{xxx} – at p<0.001 as compared to intact rats, * - at p<0.05 as compared to control rats, ** - at p<0.01 as compared to control rats, *** - at p<0.001 as compared to control rats.

Table 7

Influence of melatonin on the content of lipid peroxidation metabolites and antioxidant enzymes activity in plasma in prolonged decreased mobility stress (M+-m; n=8)

Indices / Group	Content of malonic dialdehyde in plasma mcmol/l	Content of acylhydroperoxidesiin plasma, su	Superoxidismutase activity in plasma, su	Catalase activity in plasma, mcat/ml,
Naïve (intact)	13,9±0,7	5,6±0,3	22,3±1,2	25,0±1,1
Control (stressed)	26,8±0,9^{xxx}	13,2±0,5^{xxx}	11,6±0,4^{xxx}	17,9±0,4^{xxx}
Stressed + melatonin at the dose 0,2 mg/kg	17,1±0,9***	8,2±0,5***	14,7±0,8**	19,8±0,7
Stressed + melatonin at the dose 1,0 mg/kg	16,0±0,7***	7,7±0,6***	18,4±1,1***	22,9±1,1**

Note: ^{xx} – at p<0.01as compared to intact rats, ^{xxx} – at p<0.001as compared to intact rats, * - at p<0.05 as compared to control rats, ** - at p<0.01 as compared to control rats, *** - at p<0.001 as compared to control rats.

The intraperitoneal injections of melatonin at the dose 0,2 mg per 1 kg of b.w. within 7 days of stress modeling decreased significantly the content of MDA (by 36,3%) and AHP (by 37,6%) (p<0,001). But these values were higher than the same parameters in naïve rats (p<0,05). SOD activity was increased by 20,9% compared to control group (p<0,01). Catalase activity was not changed significantly under the influence of melatonin at the dose 0,2 mg per kg b.w. However the activities of both investigated enzymes were lower than the same parameters in naïve rats (p<0,05-0,01).

The antioxidant effects of melatonin at the dose 1,0 mg per kg of b.w. were stronger than those in lower dose. The concentration of LPO metabolites were significantly less compared to control group: MDA – by 40,3%, AHP – by 41,9%. The activities of antioxidant enzymes were higher than in the control group: SOD – by 58,6% (p<0,001), catalase – by 22,5% (p<0,01). It's important that there were no differences in enzymes activities between naïve animals and ones, treated by melatonin at the dose 1,0 mg per kg b.w.

As in the liver tissue the modeling of 6 hour immobilization stress was accompanied by the increase of MDA and AHP concentrations in plasma, but the changes in the antioxidant enzyme activity had opposite directions: SOD – was increased and catalase – was decreased within 4 days after stress modeling.

The administration of melatonin at the both investigated doses suppressed LPO and the pharmacological effects of the preparation at the dose 1,0 mg per kg b.w. was significantly more expressed. If the decrease of MDA only was revealed after the injections of the dose 0,2 mg per kg b.w., the drop of both MDA and AHP concentration was observed in melatonin-treated animals at the dose 1,0 mg per kg b.w. Besides the content of LPO metabolites in this group on the fourth day after acute stress was approximately equal to that one in naïve rats.

The modeling of prolonged decreased mobility stress caused significant increase of cholesterol, TG, NEFA concentrations (by 70,2%, 42,6%, 174,6% correspondently, p<0,01-0,001) (Table 8). The administration of melatonin at the dose 1,0 mg per kg b.w. is accompanied with the drop of the content of: cholesterol – by 30,6% compared to control group. Melatonin injections at the dose 0,2 mg per kg b.w. caused less effects.

Table 8.

The influence of melatonin on the content of cholesterol, triglycerides, nonesterified fatty acids and lipoprotein of different density in plasma of rats, exposed to prolonged decreased mobility stress. (M+-m; n=8)

Index / Group	Cholesterol mmol/l	TG, mmol/l	LPVLD, mmol/l	LPLD, mmol/l	LPHD, mmol/l	NEFA, mmol/l
Naïve (intact) group	2,08±0,08	1,69±0,11	0,06±0,01	0,06±0,01	0,27±0,02	0,67±0,04
Control (stressed) group	3,56± 0.09^{xxx}	2,44± 0,11 ^{xx}	0,15±0,01 ^{xxx}	0,16±0,01 ^{x}	0,17±0,01 ^{xx}	1,98±0,10 ^{xxx}
Stressed group + melatonin at the dose 1,0 mg/kg	2,47±0,09***	1,97±0,09**	0,14±0,01	0,14±0,01	0,28±0,03**	1,32±0,05***
Stressed group + melatonin at the dose 0,2 mg/kg	2,80±0,10***	2,15±0,10	0,15±0,01	0,15±0,01	0,25±0,03*	1,40±0,08**

Note: ^{x} – at p<0.05 as compared to intact rats, ^{xx} – at p<0.01as compared to intact rats, ^{xxx} – at p<0.001 as compared to intact rats, * - as p<0.05 as compared to control rats, ** - at p<0.01 as compared to control rats, *** - at p<0.001 as compared to control rats.

The changes in the content of different types of LP were revealed in animals, exposed to prolonged decreased mobility stress: LPVLD and LPLD concentrations were increased by 166,7% and 150,0% correspondently (p<0,001), but LPHD one was less by 40,7% (p<0,01).

Melatonin-treated animals (at the both doses) were characterized by higher concentration of LPHD than in control group, but there were no differences in the content of LPVLD and LPLD.

The modeling of 6 hour immobilization stress caused the increase of the concentrations of: cholesterol – by 54,4% (p<0,001), TG – by 38,0% (p<0,01), LPVLD – by 36,0% (p<0,001), LPLD – by 61,9% (p<0,001), NEFA – by 136,7% (p<0,001) 39 hours after immobilization (Table 9). LPHD concentration was not significantly changed. The high content of NEFA remained higher than in the naïve group (by 49,4%, p<0,05). The increase of LPHD concentration was revealed 4 day after stress modeling.

Table 9

The influence of melatonin on lipid metabolism indices at the different periods after 6-hour immobilization (M+-m; n=8)

Index / Group	The period after immobilization	Cholesterol mmol/l	TG, mmol/l	LPVLD, mmol/l	LPLD, mmol/l	LPHD, mmol/l	NEFA, mmol/l
Naive (intact) group		2,04±0,02	1,79±0,12	0,25±0,02	0,21±0,02	1,20±0,05	0,79±0,07
Control (stressed) group	39 h.	3,15±0,16^{xxx}	2,47±0,10^{xx}	0,34±0,03^{xxx}	0,34±0,03^{xxx}	1,19±0,03	1,87±0,09^{xxx}
	4 days	2,19±0,05	2,01±0,11	0,20±0,02	0,19±0,02	1,35±0,05	1,18±0,09^{x}
	7 days	1,88±0,09	1,73±0,13	0,17±0,01^{xx}	0,16±0,01	1,17±0,05	0,79±0,09
Stressed group + melatonin at the dose 0,2 mg/kg	39 h.	2,91±0,19	2,31±0,17	0,35±0,04	0,34±0,04	1,39±0,06*	1,91±0,10
	4 days	2,16±0,11	1,61±0,17	0,21±0,02	0,22±0,03	1,03±0,08*	0,92±0,09
	7 days	1,90±0,15	1,91±0,15	0,20±0,02	0,19±0,02	1,17±0,06	0,69±0,11
Stressed group + melatonin at the dose 1,0 mg/kg	39 h.	2,77±0,13	2.22±0,13	0,34±0,03	0,35±0,03	1,52±0,08**	1,79±0,09
	4 days	2,08±0,09	1,72±0,15	0,26±0,02	0,26±0,02*	1,27±0,06	0,76±0,06**
	7 days	2,06±0,11	1,76±0,09	0,20±0,02	0,21±0,02	1,55±0,04***	0,74±0,07

Note: ^{x} – at p<0.05 as compared to intact rats, ^{xx} – at p<0.01 as compared to intact rats, ^{xxx} – at p<0.001 as compared to intact rats, * - at p<0.05 as compared to control rats, ** - at p<0.01 as compared to control rats, *** - at p<0.001as compared to control rats.

The administration of melatonin at the both investigated doses had no significant influence on these indices. The increase of LPHD content was revealed 39 hours after the experiment beginning.

Discussion

The results of our investigation proove the recently obtained evidence which illustrates the LPO activation in different tissues under the stress influence [1]. We found the accumulation of MDA and AHP in gingival tissue, liver tissue and plasma of rats after immobilization stress. The obtained results demonstrate different resistance of animals to stress. That is manifested by higher increase of LPO metabolites as well as the inhibition of antioxidant enzymes activity in investigated tissues and plasma of stress-nonresistant rats compared to stress-resistant ones. Multiple reports explain low resistance to stress in animals by insufficiency of stress-limiting mechanisms in them [21].

Some investigators point to close connection between LPO activation and changes in the chemical composition of parodontium [22]. It was established that the free and bound oxyprolin content was decreased, that means the reduction of collagen content as well as GAG, which are the main components of connective tissue matrix of gingiva.

Our investigation confirm the literature data that stress causes the development of structural disorders in the liver tissue, including the dilation of sinuses, dystrophy of hepatocytes, and simultaneously the reparative processes take place in the liver [5]. The principal mechanism stimulating the formation of hepatocytes injury is stress-induced activation of LPO [23]. It's known that the stress and hyperactivation of adrenergic mechanisms lead to the microcirculation disorders and hypoxia of tissue – the factors providing the LPO activation.

The functional disturbances in hepatocytes were established in this report manifesting by the suppression of protein and lipoprotein synthesis. It's known the main type of lipoproteins in rats is LPHD [24], which become manifest with antioxidant properties [24]. So, the stress causes the inhibition of different parts of antioxidant system: enzymes (SOD and catalase) and the scavengers of radicals.

The results of our investigation confirm the typical disorders of lipid metabolism in rats exposed to stress: TG, cholesterol, NEFA concentrations increase in plasma [24]. These effects are explained by stimulatory influence of catecholamines on adipocytes and decreased insulin secretion, that is accompanied with the mobilization of TG and esterification of NEFA due to adenylate cyclase activation [24]. These changes are the manifestations of metabolism shift from carbohydrate to lipid pathway [25]. The increase of cholesterol concentration is explained by the inhibition of its destruction in the liver due to the accumulation of LPO metabolites, which inhibit the main enzyme of cholesterol catabolism – 7-alpha-hydroxilase [26]. Stress causes the increase of NEFA concentration in plasma, which is the strongest stimulus of LPVLD and LPLD formation [24].

The decrease of general protein content 39 hours after stress modeling and albumin concentration 4 days after immobilization were established in our investigation. The drop of albumin, but not general protein was observed in animals exposed to

prolonged decreased mobility stress. We guess that the decrease of general protein 39 hours after stress modeling is the result of stress-induced proteolysis activation. The suppression of protein synthesis in the liver manifests itself by the decrease of albumin concentration.

Melatonin is known to reveal the beneficial effects to decrease the LPO activation by several ways. Melatonin is capable of directly scavenging the highly reactive radicals, including hydroxyl radical, superoxide anion radical, singlet oxygen, hydrogen peroxide [27, 28]. The administration of melatonin clearly had a greater stimulatory effect on antioxidant enzymes activity [11] and our data confirm this action on SOD and catalase activity in gingival and liver tissues.

Melatonin is also effective to inhibit the stress-induced hormonal changes [7, 29], which are the factors which initiate the LPO in stress. We guess that melatonin effects on components of gingival connective tissue are explained not only antioxidant action, but also hormonal effects particularly cortisol inhibition. It's known that cortisol is capable to inhibit collagen and proteoglycans synthesis.

The comparison of the effects of two doses of melatonin is shown in our investigation, and established the higher efficiency of 1,0 mg per kg b.w. dose against 0,2 mg per kg b.w. The administration of melatonin manifests the beneficial influence on LPO and collagen-GAG content in stress-nonresistant rats particularly, because they are insufficient in stress-limiting mechanisms.

The obtained results revealed the high effectiveness of melatonin as a stress-limiting substance and open the prospects of using of melatonin and its artificial analogues for pharmacological treatment of stress-induced disorders in parodontium and liver.

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